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Thiodiglycolic Acid A Major Metabolite of Bis(2-chloro-ethyl)ether

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16. ABSTRACT

Male rats were given a single oral dose (40mg/kg) of bis(2-chloroethyl)ether (BCEE). Less than 2% of the dose was recovered from the expired air as the unaltered parent compound during an 8-hr collection period. Urine samples representing 48 hr of collection were analyzed by gas chromatography-mass spectrometry. Methyl ester/trimethylsilyl ether derivatives of the isolated urinary acid fraction were prepared for gas-phase analysis. Two metabolites were identified in this fraction: thiodiglycolic acid (TDGA) and 2-chloroethyl β-D-glucosiduronic acid. Quantitative analysis for TDGA gave an average (seven rats) yield for 48 hr of 33 ± 11.8 mg (±SD) of TDGA/kg from a single dose of 40 mg/kg BCEE. The glucuronide of 2-chloroethanol was synthesized using rabbit liver microsomes in an incubation mixture. The matching mass spectrum of the glucuronide prepared in vitro to that identified in the urine of the rats verified 2-chloroethyl β-D-glucosiduronic acid as a metabolite of BCEE.

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Thiodiglycolic Acid: A Major Metabolite of Bis(2-chloroethyl)ether

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Thiodiglycolic Acid: A Major Metabolite of Bis(2-chloroethyl)ether. LINGG, R. D., KAYLOR, W. H., PYLE, S. M., AND TARDIFF, R. G. (1978). *Toxicol. Appl. Pharmacol.* **47**, 23–34. Male rats were given a single oral dose (40 mg/kg) of bis(2-chloroethyl)ether (BCEE). Less than 2% of the dose was recovered from the expired air as the unaltered parent compound during an 8-hr collection period. Urine samples representing 48 hr of collection were analyzed by gas chromatography–mass spectrometry. Methyl ester/trimethylsilyl ether derivatives of the isolated urinary acid fraction were prepared for gas-phase analysis. Two metabolites were identified in this fraction: thiodiglycolic acid (TDGA) and 2-chloroethyl β-D-glucosiduronic acid. Quantitative analysis for TDGA gave an average (seven rats) yield for 48 hr of 33 ± 11.8 mg (±SD) of TDGA/kg from a single dose of 40 mg/kg BCEE. The glucuronide of 2-chloroethanol was synthesized using rabbit liver microsomes in an incubation mixture. The matching mass spectrum of the glucuronide prepared *in vitro* to that identified in the urine of the rats verified 2-chloroethyl β-D-glucosiduronic acid as a metabolite of BCEE.

Recently, a survey conducted by the Environmental Protection Agency found bis(2-chloroethyl)ether (BCEE) in 13 of 113 municipal drinking water supplies (Dressman *et al.*, 1977). Previously, the source of this material was believed to be the chlorohydrin synthesis of ethylene oxide, but his process has not been used since 1972 (Durkin *et al.*, 1975). Since BCEE had been identified earlier in drinking water (Kleopfler and Fairless, 1972) and in surface waters (Rosen, *et al.* 1963), either there are other unknown sources of BCEE or it is persistent in the environment.

Bis(2-chloroethyl)ether is structurally related to the potent biological alkylating agents bis(chloromethyl)ether and bis(2-chloroethyl)sulfide. Biological activity has been reported for BCEE. Innes *et al.* (1969) reported apparent carcinogenicity for BCEE when given orally to mice. Simmon *et al.* (1977) reported that BCEE was mutagenic in the Ames *Salmonella* microsome assay. BCEE is relatively toxic having an oral LD50 of 75 mg/kg in rats (Christensen *et al.*, 1976). The persistence of BCEE in the environment coupled with its chemical and biological properties suggests that the biochemical fate of BCEE in mammals would be of interest in assesssing its toxicological properties. The objective of this study was to identify the major *in vivo* metabolites of BCEE so that some understanding of this compound's apparent biological activity could be obtained.

METHODS

Compound. A commercial grade (Eastman No. 886) of bis(2-chloroethyl)ether was purchased and checked for purity by gas chromatography and gas chromatography/mass spectrometry (gc/ms). The re-

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agent was chromatographed on a 5-ft-long × 2-mm-i.d. glass column packed with 0.2% Carbowax 1500 on 80/100 mesh Carbopack C (Supelco, Inc.). The helium carrier gas flow rate was set at 30 ml/min and the column was programmed from 60 to 170°C at 6°C/min. A Finnigan 9500 gas chromatograph was used with the injector and the flame ionization detector maintained at 180 and 220°C, respectively. A Varian chromatography data system was interfaced to the gc for peak area measurement and calculation of percentage composition. The reagent was found to have a purity of 98.5% and was used without further purification.

Preparation of the dose. A solution of 40 mg BCEE/ml of corn oil (Fisher laboratory grade) was prepared by volume immediately prior to the start of the dosing period. The BCEE volume was measured with a microliter glass syringe and the corn oil volume was measured with a glass pipet. The same solution was used for all dosing.

Animals and dosing procedure. Male Sprague–Dawley rats purchased from Charles River, Inc. were used throughout this study. A single dose of 40 mg/kg was administered to each rat by oral gavage with a 1.0-cc glass syringe fitted with a stainless-steel feeding needle. A total of seven rats were dosed spanning 20 calendar days. The weight range of the rats was 280 to 330 g. Control urine samples were collected for a period of 24 hr immediately preceding dosing. Animals were administered feed and water *ad libitum*.

Collection of samples. Immediately following administration of the dose, the animals were placed in Delmar–Roth metabolism cages equipped with all-glass urine–feces separators. The urine and feces traps were immersed in an ice bath. Urine samples were collected at 4, 8, 20, 32, and 48 hr. The urine samples were stored at −60°C.

Laboratory air was drawn by vacuum through the metabolism cages at a rate of 400 ml/min. The air was passed through a charcoal trap before entering the cage. To collect unaltered BCEE from the expired air, a trap was used which consisted of a 500-ml three-necked round-bottom flask fitted with a Liebig-type condenser (bottom) and a Graham-type condenser (top) in series (Fig. 1). The condensers were cooled with recirculating water at 5°C. Air, after passing through the metabolism cage, was drawn through the trap entering at one of the side necks on the flask and exiting at the top of the condenser train. Inside the trap, 250 ml of trimethylpentane (TMP) was gently refluxed.

To test the efficiency of this system, standard solutions of BCEE in pentane were placed in an open dish inside the cage and allowed to evaporate under the normal pull of air through the cage. Five runs were made in which 8 mg of BCEE (1 mg/ml pentane) was evaporated and collected over an 8-hr period. The TMP in the trap was analyzed for BCEE as described below. The recovery was 95.5 ± 1.5% (±SD) for this set of samples. Recovery over a 36-hr period was 93.8 ± 2.8% (±SD) for the same number of trials in which 24 mg of BCEE was collected per trial run. It was necessary to add TMP to the flask during the 36-hr trial period to prevent the system from going to dryness.

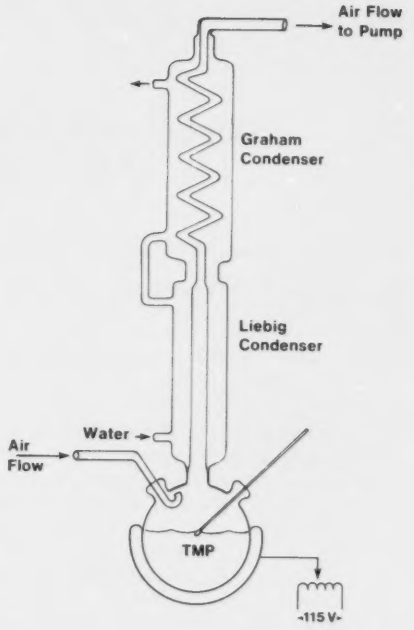

FIG. 1. Diagram of apparatus used to collect unaltered bis(2-chloroethyl)ether from the expired air. The 500-ml three-necked flask contains 200 ml of trimethylpentane gently refluxing. Air coming from the glass-enclosed metabolism cage enters at the left-side neck and exits at the top of the condenser train.

Analysis for BCEE in expired air samples. Two samples were taken from the expired air trap: (1) a sample for the 8-hr collection period immediately following dosing and (2) a sample at 48-hr representing collection between 8 and 48 hr. A Finnigan 9500 gas chromatograph equipped with a Hall electrolytic conductivity detector (Tracor Model 310) in the halide-specific mode was used for the analysis of BCEE collected in the trap. The column was 5-ft-long × 2-mm-i.d. glass packed with 3% SP 2100 on 100/200 mesh Supelcoport (Supelco, Inc.). The helium carrier gas flow rate was 30 ml/min. Samples were run isothermally at 80°C with the injector at 150°C. This system had a minimum detectable limit of 0.5 μg/ml for BCEE.

Preparation of urine samples for gc/ms analysis. The urine fractions collected at the intervals given above were thawed and combined to give a 48-hr composite sample for each rat. This sample was thoroughly mixed and 1-ml aliquots were taken for analysis. Urinary organic acids were extracted from the urine using a DEAE-Sephadex anion-exchange column with pyridinium acetate as the eluant. Preparation of the anion-exchange column, extraction, and elution of the urinary organic acids are described in numerous articles (e.g., Chalmers and Watts, 1972). To the final eluate which consisted of 25 ml of 1.5 N aqueous pyridinium acetate, 10 mg of methoxyamine hydrochloride was added. The solution was allowed to stand for 24 hr at room temperature which resulted in the conversion of all oxoacids to the corresponding methoxime derivatives. At the end of this 24-hr period, the samples were lyophilized to remove all traces of moisture. The residue was taken up in 5 ml of methanol and transferred to a reaction vial where the methanol was reduced in volume to 2 ml with a stream of dry nitrogen. Three milliliters of ethereal diazomethane (prepared using Aldrich Diazald kit) was added to this solution and allowed to stand 5 min, immediately after which the sample was evaporated to near dryness with a stream of dry nitrogen. A detailed description of methylation procedure is given by Dalgliesh *et al.* (1966). Following evaporation to near dryness, 0.5 ml of silylation-grade pyridine (Pierce Chemical Co.) and 0.5 ml of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Pierce Chemical Co.) were added and the samples placed on a heating block at 60°C for 1 hr. The net result of these steps is to convert all carboxylate groups to methyl esters (Me-ester) and all hydroxyl groups to trimethylsilyl ethers (TMS-ethers).

After completion of the preparative steps for gc/ms analysis, the headspace was purged with dry nitrogen, and the vials were sealed with crimped on Teflon-backed septa (Pierce Chemical Co.). Samples were analyzed by gc/ms immediately after preparation.

Gas chromatography/mass spectroscopy analysis. All mass spectra were obtained at 70 eV on a Finnigan Model 3300 quadrupole mass spectrometer interfaced to a Finnigan Model 9500 gas chromatograph (Finnigan Corp). Mass spectra were acquired continuously at unit resolution and recorded on a disk by a System 150 (System Industries)-automated gc/ms data system. Integration time was varied from low to high masses: 40 to 220 atomic mass units (amu) scanned at 4 msec/amu, 221 to 320 amu at 7 msec/amu, and 321 to 520 amu at 10 msec/amu. The gc column was 5-ft-long × 2-mm-i.d. glass packed with 100/120 mesh Ultra-Bond 20 M (RFR Corp) and was programmed from 90 to 225°C with a 4-min isothermal hold at the beginning of the run. The injector and separator of the gc/ms were both at 230°C. Two microliters of sample was injected.

Quantitative analysis for TDGA. Because of the superior chromatographic characteristics of the trimethylsilyl ester (TMS-ester) of TDGA over that of the Me-ester, this derivative was prepared for the quantitative analysis of TDGA. One-milliliter urine samples were prepared exactly as described by Thompson and Markey (1975) which resulted in trimethylsilylation of all active hydrogens (TMS-ester/TMS-ether). A Finnigan 9500 gc equipped with a flame ionization detector and a 5-ft-long × 2-mm-i.d. glass column packed with 10% SP 2100 on 100/120 mesh Supelcoport (Supelco, Inc.) was used for the quantitative analysis. The gc was programmed from 90 to 280°C following a 4-min isothermal hold, and the injector and detector were at 280 and 320°C, respectively. The helium carrier gas flow rate was set at 30 ml/min. Peak areas were measured with a Varian Chromatography data system interfaced to the gc. Thiodiglycolic acid was purchased 98% pure from Aldrich Chemical Co. and used without further purification. The quantity of TDGA in the urine samples was determined by extrapolation from standard curves. Control urine was spiked with known amounts of TDGA and subjected to the same preparation steps as the authentic samples. For seven standard samples of 100 μg of TDGA/ml of urine, a recovery of 94.1 ± 3.0% (± SD) was obtained. No TDGA was found in the control urines.

Preparation of the dimethyl ester of TDGA. The dimethyl ester of TDGA was prepared by methylation of TDGA with diazomethane. A standard solution containing 0.1 mg/ml TDGA in methanol was prepared. To a 1-ml aliquot of this solution, 2 ml of an etheral diazomethane (Aldrich Diazald kit) solution was added and allowed to stand 5 min after which the solution was immediately reduced to near dryness with a stream of dry nitrogen. After evaporation, 0.5 ml of silylation-grade pyridine and 0.5 ml of BSTFA were added to the residue in order to provide a solution for gc/ms injection which matched that of the urine samples. The Me-TDGA standard was analyzed by gc/ms in exactly the same manner described for the urine samples.

Preparation of 2-chloroethyl β-D-glucosiduronic acid (CEGA). Pellets of rabbit and rat liver microsome fractions were prepared according to standard procedures (Mazel, 1971). Animals were not pretreated and were sacrificed following a 24-hr fast. The livers were perfused *in situ* with isotonic potassium chloride (1.15% KCl). Five-gram fractions of liver were taken and homogenized with isotonic KCl utilizing a Potter–Elvehjem glass tube and Teflon pestle. The homogenate was spun at 10,000*g* for 30 min. The supernatant was decanted and spun at 100,000*g* for 60 min. After completion of centrifugation, the super-

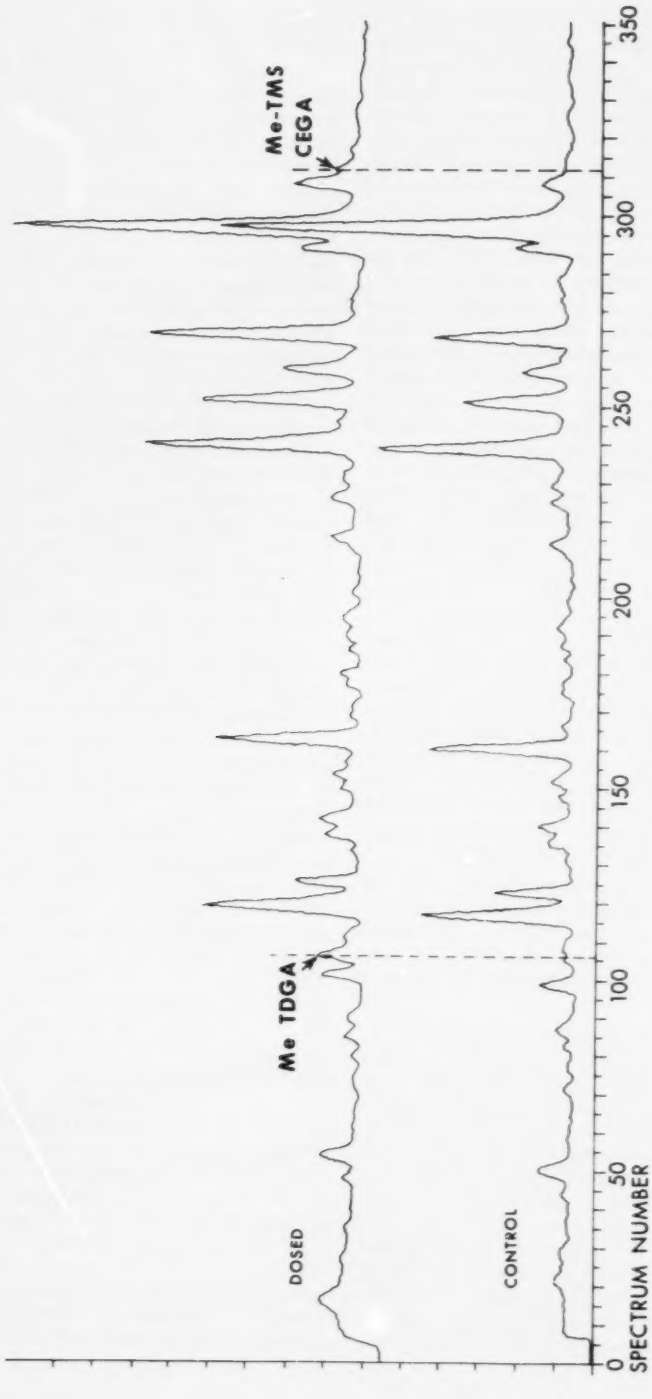

FIG. 2. Reconstructed gas chromatograms from gas chromatography/mass spectrometry runs of derivatized urinary acid fractions of normal rat urine (lower) and of a 48-hr composite urine sample (upper) from a rat which received a 40 mg/kg oral dose of bis(2-chloroethyl)ether.

natant was withdrawn and the pellets were left in the centrifuge tubes and stored at −60°C until used.

The microsome pellets were resuspended in 5 ml of 50 mM Tris–HCl buffer (pH 7.40) immediately before use. The procedure outlined by Lucier *et al.* (1971) was used for preparation of the incubation mixture which consisted of 0.5 ml of UDPGA solution (3.89 mg of UDPGA/ml of 50 mM Tris–HCl buffer, pH 7.40), 1 ml of the liver microsomal solution, and 3.5 ml of same Tris–HCl buffer in a 50-ml Erlenmeyer flask. The aglycon, 2-chloroethanol (0.1 ml) was added directly to the incubation mixture. The flask was covered and placed in a metabolic stirrer for 2 hr at 37 to 40°C.

Separation and gc/ms analysis of CEGA. Following the incubation period, the reaction mixture was transferred to a chilled centrifuge tube and centrifuged at 32,200 rpm for 30 min in a Sorvall RC2-B refrigerated ultracentrifuge after which the supernatant was decanted and the pH was adjusted, if necessary, to 7 to 8 with 0.1 M NaOH. The glucuronide was extracted from the supernatant using the same procedure described above for the extraction of the urinary organic acids.

Following elution of the glucuronide with 1.5 M aqueous pyridinium acetate, the solution was lyophilized to dryness and the residue taken up in methanol. The methanol was reduced in volume to 1 ml with a stream of dry nitrogen. A Me-ester/TMS-ether derivative of the glucuronide was prepared using the same reagents and conditions described for the urinary acids. In order to aid in the interpretation of the mass spectrum, another sample was prepared in which *N,O*-bis(perdeuterotrimethylsilyl)trifluoroacetamide (BSTFA-d_{18}, Regis Chemical Co.) was substituted for BSTFA. This reaction produced the corresponding TMS-d_0-ether derivatives.

RESULTS

The amount of unaltered BCEE collected from the expired air was determined at 8 and 48 hr following dosing. The 0- to 8-hr samples had no more than 1 μg of BCEE/ml of TMP determined by gc analysis. The data could not be statistically treated because of the low concentrations obtained and the minimum detectable amount of 0.5 μg of BCEE/ml TMP. BCEE was not detectable in the 8- to 48-hr samples.

A figure representing the upper limit of BCEE expired unaltered following dosing can be made if the value of 1 μg of BCEE/ml of TMP is taken as the maximum recovery of BCEE from the expired air for a 0-to 48-hr collection period. Multiplying this figure by the adjusted final TMP volume of 200 ml (during a run, TMP was lost from the trap), and then dividing by the average total BCEE dose of 12.7 mg, a value of 1.6% BCEE recovered unaltered is obtained.

Figure 2 represents typical gc/ms runs of Me-ester/TMS-ether (Me-TMS)-derivatized

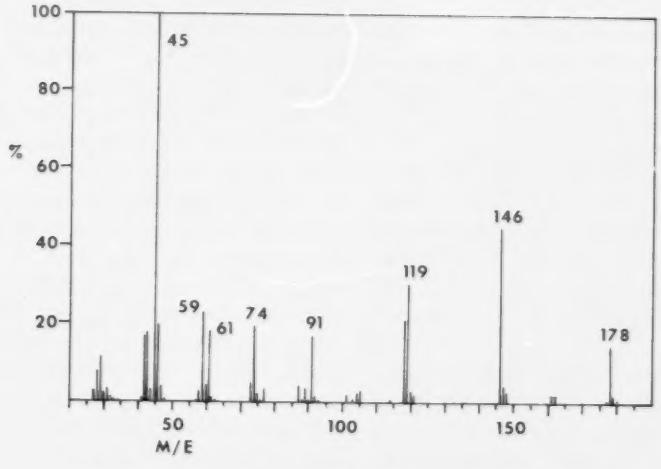

FIG. 3. Mass spectrum of the peak labeled Me-TDGA in Fig. 2.

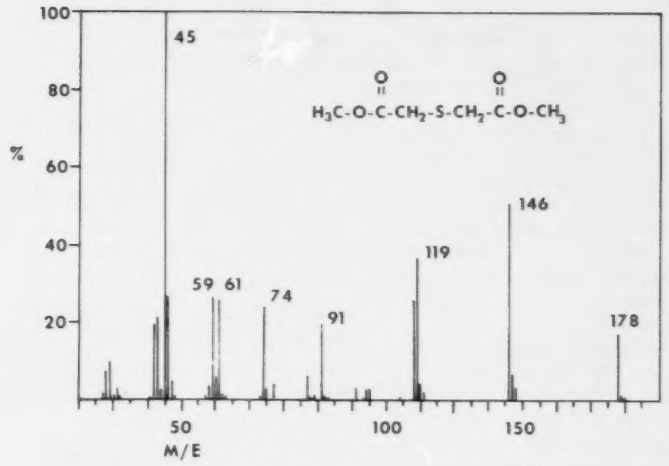

FIG. 4. Mass spectrum of the dimethyl ester of thiodiglycolic acid.

urine samples; the lower curve represents a 24-hr control urine sample taken immediately prior to dosing and the upper curve represents the profile of a composite urine sample taken over 48 hr following dosing. Identification of possible metabolites was accomplished by comparison of peak heights and spectra between the dosed and control samples. One peak appearing in all the urine samples of the dosed animals and which was not identifiable in any of the control samples is labeled as Me-TDGA in Fig. 2. The mass spectrum of this peak (Fig. 3) was identified as that of the dimethyl ester of TDGA. A comparison of gc retention times and the mass spectrum (Fig. 4) of a methylated standard of TDGA confirmed the identification.

Quantitative analysis by gc for TDGA as the trimethylsilyl ester of TDGA (TMS-TDGA) gave an average recovery for 48 hr of 33.0 ± 11.8 mg ($\pm$SD) of TDGA/kg following a single oral dose of 40 mg/kg BCEE. The identity of the TMS-TDGA was confirmed by gc/ms.

Another peak which consistently appeared in the urine sample of the dosed rat is the shoulder peak labeled Me-TMS CEGA in Fig. 2. This peak has been identified as the Me-TMS derivative of CEGA from its mass spectrum which is given in Fig. 5. Fragment ions (m/e) such as m/e 204 and 217 which are characteristic of TMS-derivatized sugar moieties are present. The appearance of this compound in the urinary acid fraction suggested the presence of a carboxylate group (some neutral compounds are known to be extracted from urine by DEAE-Sephadex anion exchange).

Billets *et al.* (1973) have evaluated the mass spectra of a number of Me-TMS-derivatized glucuronides of ether-linked drugs. One feature which strongly influences the overall mass spectral pattern is whether the ether link to the aglycon is through an aliphatic or aromatic hydroxyl group. The examples of aliphatic ether-linked glucuronides given by these authors are those for which the aglycon is a large drug moiety and, therefore, may not be representative of the general case when, for instance, the aglycon is a short-chain aliphatic alcohol. Jaakonmaki *et al.* (1967) published the spectrum of the Me-TMS derivative of ethyl β-D-glucosiduronic acid. This is the only example found in the literature where the aglycon is a small-chain aliphatic alcohol, so no general features of these spectra are yet described.

A glucuronide of 2-chloroethanol would

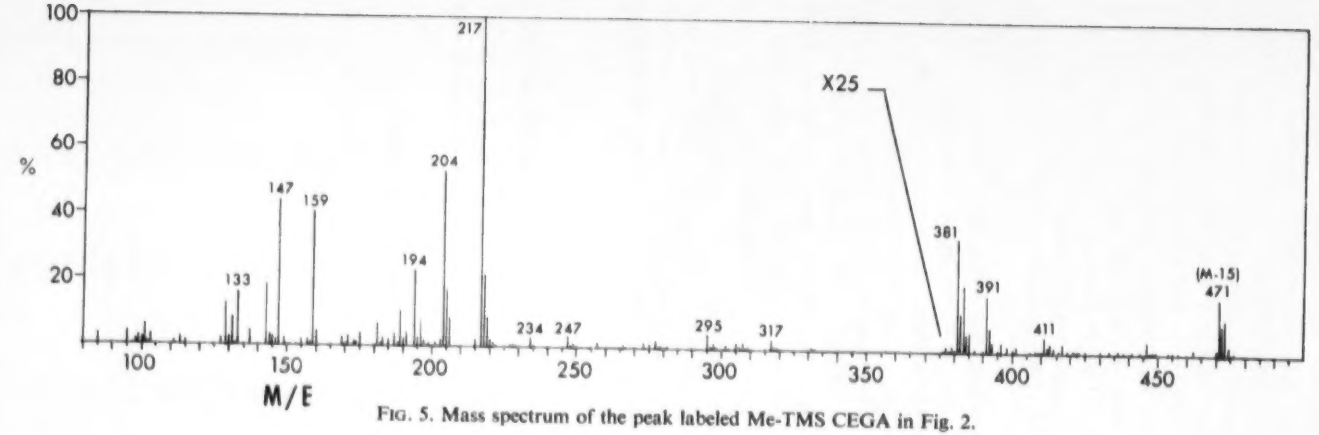

FIG. 5. Mass spectrum of the peak labeled Me-TMS CEGA in Fig. 2.

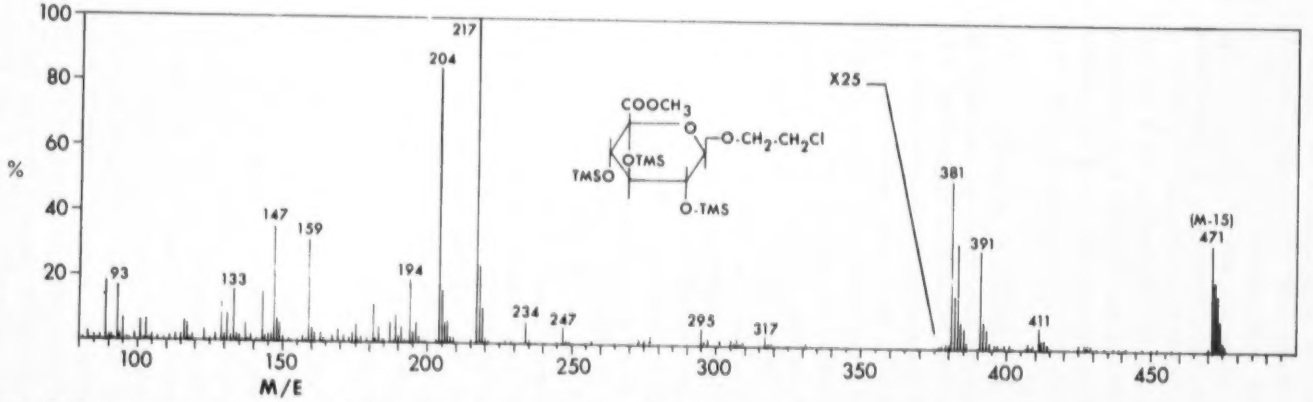

FIG. 6. Mass spectrum of the methyl-ester/trimethylsilyl-ether derivative of 2-chloroethyl β-D-glucosiduronic acid obtained by incubating 2-chloroethanol with rabbit liver microsomes and UDPGA in Tris–HCl buffer.

have a nominal molecular weight of 486 amu. Molecular ions, in general, are not observed for trimethylsilylated glucuronides. Instead, the largest mass usually observed is M-15 (M-CH_3) which is at *m/e* 471 (486-15) in Fig. 5. Another ion characteristic of glucuronides is M-105 [M-(CH_3+$(CH_3)_3$SiOH)] which is found at *m/e* 381 (486–105) in Fig. 5. An *m/e* which distinguishes aliphatic from aromatic ether-linked glucuronides is M-191 (Billets *et al.*, 1973). This peak is at *m/e* 295 (486–191) in Fig. 5.

In order to be able to completely characterize a glucuronide, those ions containing all or part of the aglycon must be identified. This may be accomplished by high-resolution mass spectrometry. Since this facility was not available, glucuronides of 2,2-dichloroethanol and 1,3-dichloro-2-propanol were prepared in the same manner as described for 2-chloroethanol. These compounds would have the same glycon–aglycon linkage as 2-chloroethanol while providing easily recognizable mass shifts through the chlorine multiplets. The same *m/e* values, such as M-15 and M-105, which normally appear in the mass spectra of Me-TMS glucuronides, were observed. The M-191 peak characteristic of ether-linked aliphatic glucuronides was also observed in each case. In addition, a peak was observed at M-292 for each glucuronide prepared. For CEGA, the M-292 peak appears at *m/e* 194 (Fig. 5). Close examination of *m/e* 194 to 196 in Fig. 5 reveals that a single chlorine multiplet might exist at these masses.

The mass spectrum of the Me-TMS derivative of CEGA prepared *in vitro* with rabbit liver microsomes is given in Fig. 6. A close match to that in Fig. 5 is made. Although the glucuronide was not isolated in its pure form from the microsomal preparation, it was rendered "chromatographically" pure by the gc column before the spectrum of the glucuronide was recorded. Figure 7 is the chromatogram from a gc/ms run of the derivatized CEGA extracted from the microsomal preparation. The retention time of the peak labeled Me-TMS CEGA matches that of the peak labeled Me-TMS CEGA in Fig. 2.

The mass spectrum of a Me-ester/TMS-d_9-ether (Me-TMS-d_9) derivative of CEGA prepared by microsomal synthesis was obtained. Table 1 lists the mass shifts from Me-TMS to Me-TMS-d_9 which serve to confirm the identity of the spectrum in Fig. 5 as that of Me-TMS CEGA. The shift of *m/e* 471 by 24 mass units verifies the nominal molecular weight assignment at 486 amu. A Me-TMS derivative of CEGA would have three TMS groups on the glycon moiety. Therefore, the mass shift from tri-TMS to tri-TMS-d_9 would be 27 amu which would give a nominal molecular weight to Me-TMS-d_9 CEGA of 513 amu (486+27). A loss of $-CD_3$ from the molecular ion gives a peak at M-18 of *m/e* 495 which is 24 amu above *m/e* 471, the M-CH_3 peak for the Me-TMS derivative. The M-105 (*m/e* 381) peak is shifted to *m/e* 396 corresponding to the loss of 117 amu [M-(CD_3+$(CD_3)_3$SiOH)] from the Me-TMS-d_9 derivative. High-resolution mass spectrometry has been used to confirm the empirical formula of the M-191 peak for

TABLE 1

MASS SPECTRAL DATA FOR Me-TMS AND Me-TMS-d_9 DERIVATIVES OF 2-CHLOROETHANOL β-D-GLUCOSIDURONIC ACID

Me-TMS		Me-TMS-d_9	
m/e	Relative intensity (%)	*m/e*	Mass shift
486	0	513	27
471	1.3	495	24
411	0.3	435	24
391	1.2	415	24
381	2.0	396	15
317	3.5	335	18
295	5.3	313	18
247	5.2	265	18
234	6.7	252	18
217	100.0	235	18
204	84.2	222	18
194	19.3	203	9
159	31.6	165	6
147	35.5	162	15

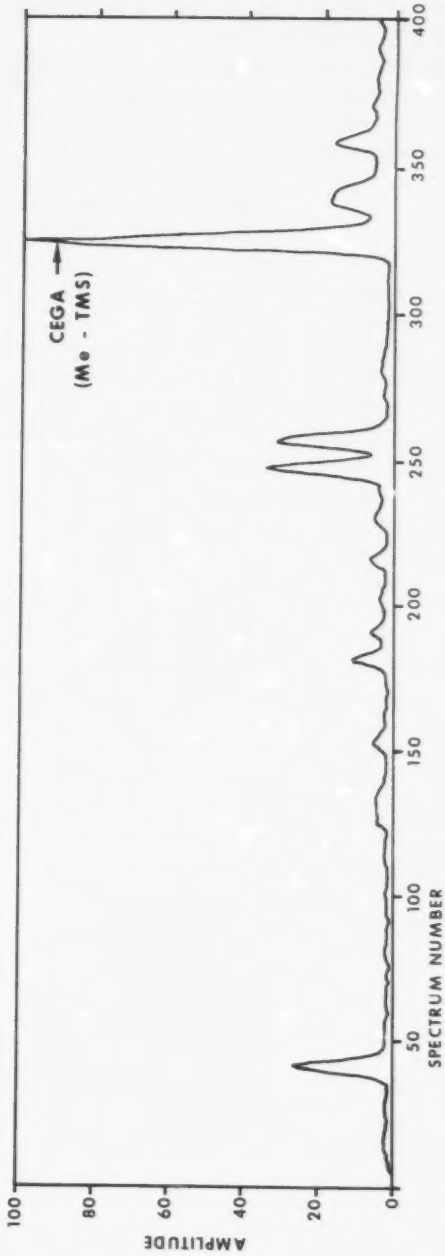

FIG. 7. Reconstructed gas chromatogram of acid fraction extracted from incubation mixture to which 2-chloroethanol had been added. The mass spectrum of the peak labeled Me-TMS CEGA is that given in Fig. 6.

glucuronides (Billets *et al.*, 1973). This peak involves the loss of one complete TMS group in addition to parts of the glycon. A shift of *m/e* 295 to *m/e* 313 upon substituting TMS-d_9 is a difference of 18 amu which is in agreement with the loss of a fragment containing one TMS group from the parent compound. The peak at M-292 is shifted by 9 amu which suggests that the moiety lost from the parent contains two TMS groups.

DISCUSSION

The best studied examples on the metabolic fate of dialkyl ethers are those of the volatile anesthetics. Van Dyke *et al.* (1964) administered [1-^{14}C]-diethyl ether to rats by intraperitoneal injection and recovered 4% of the administered dose as CO_2 in 24 hr and 2% as nonvolatile metabolites in the urine. Onchi and Asao (1961) administered unlabeled diethyl ether to humans and recovered 37 to 84% as the unaltered ether over a 2-hr period. Eighty-five to ninety percent of [^{14}C]-methoxyflurane (2,2-dichloro-1,1-difluoroethyl methyl ether) was excreted by rats unchanged within 30 hr following intraperitoneal injection (Van Dyke *et al.*, 1964). Holaday *et al.* (1970) administered [^{14}C] methoxyflurane to patients undergoing surgery and to one healthy subject. Approximately 28% of the estimated absorbed dose given to the healthy subject was recovered in a 48-hr period as the unaltered compound. In contrast, the results of this study demonstrated that less than 2% of a 40 mg/kg oral dose of BCEE was exhaled unaltered during the initial 8 hr following injection and, between 8 and 48 hr BCEE was not detectable in the expired air by the method employed. The different routes of administration used in each of the mentioned studies makes attaching any significance to this comparison hypothetical. One might guess that volatility partially accounts for these differences in the excretion patterns, but other factors such as dose levels also contribute which is evident in the example of vinyl chloride (VC): A single oral dose of 1 mg/kg VC fed to rats resulted in only 2% excreted unchanged in 72 hr while 67% was excreted unchanged at the 100 mg/kg dosage level (Watanabe *et al.*, 1976).

The evidence does point to the fact that a significant proportion of BCEE was absorbed into the body and excreted as the urinary metabolite TDGA. Because the BCEE was not labeled, the proportion of the parent compound converted to TDGA can be calculated only if the exact stoichiometric relationship is known. Two possibilities can be suggested: First, if one molecule of TDGA resulted theoretically from each BCEE molecule, then the percentage conversion of BCEE to TDGA in 48 hr is calculated to be 77.6% with a SD of ±23.7%; second, if a 2 : 1 (a yield of two molecules of TDGA per molecule of BCEE) relationship existed, then the result is 38.8% conversion with a SD of ±11.8%. These values were calculated using the average dose of BCEE (12.7 mg) and the average recovery of TDGA (33.0 ± 11.8 mg).

The appearance of TDGA in the urine suggests that BCEE or its metabolites conjugate with free sulfhydryl groups. A mechanism that could be postulated would be cleavage of the ether linkage to form an intermediate containing two carbons and possibly chlorine and/or oxygen which then reacts with free sulfhydryl groups. Examples of compounds which have been shown to give TDGA as a major metabolite are 2-chloroethanol, chloroacetic acid, and vinyl chloride. Johnson (1967) isolated the precursor of TDGA, 2-carboxymethyl glutathione, from the livers of rats fed 2-chloroethanol. Yllner (1971) isolated TDGA as the major metabolite from the urine of white mice fed chloroacetic acid. Seventy-two-hour composite urine samples from rats receiving orally 0.05, 1.0, and 100 mg/kg vinyl chloride contained TDGA which, in each case, accounted for roughly 25% of the radioactivity collected in the urine at each dosage level (Watanabe *et al.*, 1976).

Ether cleaving enzymes have been demonstrated to exist in the liver microsomes for methyl-aryl ethers (Axelrod, 1956) and methyl-haloalkyl ethers such as methoxyflurane (Van Dyke and Chenoweth, 1965). Data on the biotransformation of dialkyl ethers where both alkyl groups are larger than methyl are lacking in the literature. The difference in the percentage of BCEE appearing as a urinary metabolite compared to that of ethyl ether suggests that the chlorine substituent could lead to a more facile metabolic breakdown.

The presence of the glucuronide of 2-chloroethanol lends support to the hypothesis that some of the BCEE is cleaved at the ether linkage. The exact identity of the intermediate metabolite which leads to the conjugated-2-chloroethanol remains unknown. All attempts to isolate free 2-chloroethanol gave negative results (unpublished observations). Smith and Williams (1954) attempted to isolate the glucuronide of 2-chloroethanol from rabbits fed this compound but failed concluding that this was because of the rapid oxidation of 2-chloroethanol to 2-chloroacetic acid which is highly toxic. Free chloroacetic acid has not been found in the urine of the rats fed BCEE (Fig. 2). These workers were able to isolate glucuronides of 2,2-dichloroethanol and 1,3-dichloro-2-propanol. It is interesting to note here that no CEGA was synthesized with the rat liver microsomes while this same microsome preparation was active in the synthesis of the glucuronides of 2,2-dichloroethanol and 1,3-dichloro-2-propanol. Glucuronides of all three chlorinated alcohols mentioned were obtained by using rabbit liver microsomes.

Another possible mechanism for the biotransformation of BCEE is direct replacement of the chlorine by sulfhydryl-containing compounds followed by metabolic degradation of the conjugated product to TDGA. Alkyl halides have been shown to be dechlorinated enzymatically but the rate at which this occurs is not enhanced by the presence of free sulfhydryl-containing compounds (Van Dyke and Wineman, 1971). Other workers (Bray *et al.*, 1952) have found that dechlorination proceeds nonenzymatically by apparent formation of a carbon–sulfur bond and liberation of chloride ion. Obviously, the biological stability of any single carbon–chlorine bond depends on the overall structure of the molecule undergoing biotransformation. Some carbon–chlorine bonds might exhibit stability toward enzymatic attack while chemically unstable carbon–chlorine bonds could undergo direct replacement of the chlorine with sulfhydryl-containing compounds. The role of intermediates must be considered here: Dehalogenation of a reactive intermediate formed through the action of enzyme systems other than those which directly dehalogenate can occur when this intermediate reacts with free sulfhydryl groups. The ether linkage to the nearest neighbor carbon of the carbon–chlorine can be expected to contribute to the overall reactivity of BCEE.

Terminal oxidation of the β-carbon containing the chlorine has been shown to occur for bis(2-chlorisopropyl)ether (BCIE) (Smith *et al.*, 1977). Recently, the amount of a single 100 mg/kg oral dose of BCIE converted to 2-(2-chloro-1-methylethoxy)propionic acid in 24 hr by the rat has been quantitated at $38 \pm 4\%$ ($\pm$SD) (unpublished results). An analogous compound was not identified in the urine of the rats dosed with BCEE (Fig. 2).

The amount of BCEE converted to CO_2 can only be determined with the use of ^{14}C-labeled BCEE. This work is now in progress.

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